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Prevention of Autoimmune Disease by Retroviral-Mediated Gene Therapy[1]

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T lymphocytes have been implicated in a variety of autoimmune diseases, and therefore one potential therapeutic approach would be to tolerize the pathogenic self-reactive T cells. In this study, we examined whether retroviral gene therapy could be used to induce tolerance and prevent autoimmunity using a transgenic mouse model for experimentally induced diabetes. In this model, the lymphocytic choriomeningitis virus (LCMV) glycoprotein (gp) is expressed on the β-islet cells of the pancreas under the control of the rat insulin promoter (RIP). Previous work showed that the T cells specific for the gp remain unaware of the transgenic gp Ag expressed by the islet cells, and infection with LCMV leads to immune-mediated diabetes. To tolerize the gp-specific pathogenic T cells, a retroviral vector (RV) expressing the LCMV gp was constructed, RV-gp. Replication-defective recombinant retroviruses were used to transduce bone marrow cells, which were subsequently infused into host RIP-gp transgenic animals. Unlike control animals, RV-gp chimeric animals did not possess T cells specific for the gp Ag as measured by proliferation and cytotoxic function, and further analysis suggested that tolerance of the gp-specific self-reactive T cells occurred by clonal deletion. Further experiments demonstrated that chimeric RIP-gp transgenic animals generated using bone marrow transduced with RV-gp did not develop experimentally induced diabetes. Our animal model demonstrates that retroviral gene therapy may cure immune-mediated diabetes by providing long lasting Ag-specific tolerance. *The Journal of Immunology,* **1995, 155: 5404–5408.**

Autoimmune disease is often mediated by T cells that have not received tolerogenic signals to sequestered self Ags (1, 2). Several natural animal models of experimentally induced and spontaneous autoimmunity, as well as transgenic models, have been studied to further understand how these T cells escape self-tolerance mechanisms and become activated to induce immunopathologic destruction (3–10). An ideal approach to treating autoimmune disease would be to manipulate the T cell repertoire of the host so that auto-Ag-specific tolerance can be induced. In the past, retroviral vectors (RVs) have been used to attempt to eliminate superantigen or alloreactive T cells (11, 12). Another possible goal would be to tolerize T cells of defined specificity and eliminate autoimmune disease in a defined model.

Gene therapy has been considered an approach for treating a broad spectrum of genetic disorders and cancer, as well as cardiovascular disease and AIDS (13–15). Delivery of the gene may be accomplished by several techniques, however the majority of studies have employed RVs for gene transfer. Replication-incompetent retroviruses may be generated at high titers that can efficiently transduce a variety of cell types ex vivo, before adoptive transfer in vivo (16–19).

In this study, we examined whether bone marrow cocultured with retroviruses expressing a defined self Ag could generate specific T cell tolerance in reconstituted chimeric animals. Two basic mechanisms of T cell tolerance have been defined, clonal deletion and clonal inactivation (20). Several studies have shown that T cell tolerance may be readily induced against determinants expressed by bone marrow-derived cells (21–23). We therefore attempted to determine whether bone marrow cells that were genetically modified by retroviral gene delivery were sufficient to induce tolerance against a specific autoantigen and ultimately prevent experimentally induced diabetes.

We have previously described a transgenic mouse model, RIP-gp[3] that expresses the lymphocytic choriomeningitis virus gp (LCMV-gp) on the β-islet cells of the pancreas. LCMV-gp-specific T cells in these mice are not tolerant to the LCMV-gp expressed on the pancreas, and on activation by infection with LCMV, the gp-specific T cells infiltrate and destroy the islets, leading to diabetes (5, 24). To tolerize the gp-specific T cells, we generated a RV that expresses LCMV-gp (RV-gp). The following studies were done with chimeric animals that were made using bone marrow transduced with recombinant retroviruses. Experiments addressed whether LCMV-gp-specific T cell tolerance could be induced, and whether immune-mediated diabetes could be prevented in our animal model.

Materials and Methods

Mice

RIP-gp (Bln line) (5) and TCR (327 line) (25) transgenic lines were bred and typed as previously described (24), in specific pathogen-free conditions according to institutional guidelines. RIP-gp animals were generated in the

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[3] Abbreviations used in this paper: RIP; rat insulin promoter, gp; glycoprotein, LCMV; lymphocytic choriomeningitis virus, RV; retroviral vector.

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C57BL/6J strain, while the TCR line has been backcrossed six times with C57BL/6J mice. Donor C57BL/6J animals were purchased from The Jackson Laboratory (Bar Harbor, ME).

Retroviral vectors

RV-gp was generated by cloning the 1.6-kb *Bam*HI LCMV-gp cDNA into the *Bgl*II site of the MSCV RV described previously (19). This vector includes variant long terminal repeats and a mutated 5' untranslated region for efficient expression in hematopoietic cells. The ψ^+ region includes sequences necessary to generate high viral titer of replication defective retroviruses. The original MSCV vector was used as a control, RV-neo.

Northern blot analysis

RNA was extracted from cell lines using the guanidine isothiocyanate method (26). Ten micrograms of total RNA were run on a 1% formaldehyde gel and transferred to a nitrocellulose filter. The positive control sample contained 0.5 μg of RNA from LCMV-infected cells with 10 μg of carrier RNA from uninfected National Institutes of Health (NIH) 3T3 cells. Filters were prehybridized in 50% deionized formamide, 5X SSPE, 0.5% SDS, 5X Denhardt's solution, and 1 mg/ml salmon sperm DNA for 4 h. ^{32}P-labeled LCMV-gp specific probe was generated (Multiprime labeling kit; Amersham, Bucks, UK), and 1 × 10^6 cpm/ml was added to the prehybridization mix for 20 h at 42°C. Filters were washed and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 18 h.

Generation of chimeric animals

Eight-week-old female C57BL/6J or TCR transgenic mice were used as bone marrow donors. Mice were injected with 150 mg/kg 5-fluorouracil and 4 days later, the bone marrow was harvested. After erythrocyte lysis in 0.17 M ammonium chloride, cells were cultured at 5 × 10^5 cells/ml in Iscove's modified Dulbecco's medium (IMDM) supplemented with 50 μM 2-ME, 10% heat-inactivated FCS (HyClone, Logan, Utah), supplemented with IL-3 and IL-6 conditioned media as previously described (27). After 48 h, bone marrow cells were cocultivated with a subconfluent monolayer of fibroblasts producing appropriate replication defective retroviral particles in the same conditioned media, which included 8 μg/ml polybrene. After a further 48 h, nonadherent bone marrow cells were harvested, centrifuged, and resuspended in the presence of 0.75 mg/ml G418 (Life Technologies, Inc., Grand Island, NY). Cells were harvested after 24 h and 5 × 10^5 to 2 × 10^6 cells were infused into irradiated recipients (900 rads) by i.v. tail vein administration. Host animals were reconstituted for 10 wk to 5 mo before further experiments were done.

Cytotoxic assays

MC57G fibroblast target cells (H-2^b) were either infected with LCMV or recombinant vaccinia viruses expressing the LCMV-gp or LCMV-np and labeled with ^{51}chromium for 2 h. Target cells (1 × 10^4) were incubated with spleen effector T cells from C57BL/6 mice infected in vivo 8 days before the assay, at various ratios in a final volume of 200 μl IMDM and 10% FCS. The cells were incubated in 96-well round-bottom plates for 4 to 5 h at 37°C, and 70 μl of the supernatant was removed and counted. Percent specific release was calculated as (cpm experimental release − spontaneous release)/(total release − spontaneous release) × 100.

Proliferation assay

Single cell suspensions were made from the spleen, and 1 × 10^5 cells were cocultured with 2 × 10^4 stimulator cells in 96-well flat-bottom plates. The peritoneal macrophages from LCMV-infected or uninfected mice that were used as stimulator cells were obtained from mice given an i.p. injection of 2 ml thioglycollate 6 days before the assay. Cells were incubated for 48 h in IMDM plus 10% FCS, 2 × 10^{-5} M 2-ME, glutamine and penicillin, and streptomycin (100 U/ml), and then 1 μCi of [^{3}H]thymidine (NEN, Boston, MA) was added to each well. After 16 h, the cells were harvested and counted on a Matrix 96 direct β-counter (Canberra Packard, Meriden, CT).

Induction of diabetes

Mice were given an i.v. infusion of 2000 plaque-forming units LCMV (Armstrong). Blood glucose levels were monitored regularly, and quantitated using a Reflolux III (Boehringer Mannheim, Laval, QC).

Detection of retroviral DNA in chimeric animals

Tissues from various organs were homogenized in PBS and incubated with 0.2 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) for 2 h at 37°C. Samples were extracted with phenol chloroform several times and then precipitated. DNA (0.5 μg) was amplified using neomycin-specific primers (CCGGTGCCCTGAATGAACTGC) (CAATATCACGGGTAGC CAACG) for 30 cycles. Ten microliters were electrophoresed on a 1% agarose gel and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). A neomycin-specific probe (3 × 10^5 cpm/ml), generated by random priming (Amersham Multiprime labeling kit), was added to a hybridization solution (5X SSC, 5X Denhardt's solution, 0.1% SDS, and 10% dextran sulfate) and incubated overnight at 65°C. After two washes in 2X SSC, 0.1% SDS, and 0.2X SSC 0.1% SDS, autoradiographs were developed after a 2-h exposure.

Results

To determine whether retroviral gene therapy could be used to alter the T cell repertoire and tolerize pathogenic T cells, a RV was constructed to express the model autoantigen LCMV-gp. The LCMV-gp cDNA was cloned in the *Bgl*II site of MSCV (RV-gp) (Fig. 1*A*). Vector DNA was transfected into a packaging cell line that provides the necessary proteins in *trans* for production of recombinant RV-gp as infectious replication-defective viral particles (28). Cell lines were selected that were capable of exporting recombinant RV-gp retrovirus at high titers (10^6 CFU/ml). Control cell lines were also established that produce a high titer of the MSCV vector (RV-neo).

To determine whether the recombinant retrovirus expressed LCMV-gp, NIH 3T3 cells were infected with RV-gp or RV-neo, and G418-resistant cell lines were established. Northern blot analysis, using a probe specific for LCMV-gp, indicated that LCMV-gp mRNA was present only in cell lines infected with LCMV or RV-gp (Fig. 1, *B* and *C*).

We examined whether RV-gp could induce tolerance of LCMV-gp-specific T cells by several criteria. Assays were done to determine whether functional LCMV-gp-specific CTL were detectable in chimeric animals. RV-gp or RV-neo transduced bone marrow was used to reconstitute irradiated RIP-gp mice (27, 29). These mice and control C57BL/6 mice were infected with LCMV and 8 days later, the spleen cells were assayed for cytotoxic function (Fig. 2). LCMV-gp-specific CTLs were not detected in chimeric mice receiving bone marrow transduced with RV-gp, whereas an efficient LCMV-gp-specific cytotoxic response was seen in control animals. Effector cells from all mice lysed target cells infected with LCMV or target cells expressing LCMV nucleoprotein (np), but did not lyse fibroblasts infected with the control vaccinia virus. This assay shows that a functional LCMV cytotoxic response is generated in chimeric animals treated with RV-gp due to the recognition of other viral epitopes such as the np. The fact that a cytotoxic response against the LCMV-gp was absent, demonstrates that T cell tolerance is highly specific and does not compromise the host repertoire against other foreign determinants.

To examine the mechanism of tolerance, we used TCR transgenic mice expressing the Vα2/Vβ8.1 TCR specific for the LCMV-gp presented in the context of H-2D^b (25). Bone marrow from TCR transgenic mice was transduced with either RV-gp or RV-neo and infused into irradiated C57BL/6 mice. The thymus and lymph nodes were analyzed 10–12 wk later for the presence of the transgenic TCR using mAbs specific for the Vα or Vβ-chains. Mature CD8$^+$ thymocytes from RV-neo control animals expressed the transgenic TCR, whereas CD8$^+$ thymocytes from RV-gp animals did not show any detectable T cells expressing the transgenic αβ heterodimer (data not shown). Figure 3*A* indicates that Vα2$^+$ CD8$^+$ and Vβ8$^+$ CD8$^+$ lymph node T cells were present in chimeric mice that were reconstituted with RV-neo transduced bone marrow, whereas less than 1% of peripheral T cells were detected in mice that were reconstituted with RV-gp transduced bone marrow. However, in mice reconstituted with RV-gp-treated bone marrow, a significant population of Vα2 and/or Vβ8.1 T cells was present that did not express the CD8 coreceptor. This demonstrates that the TCR transgenic bone marrow partially reconstituted the

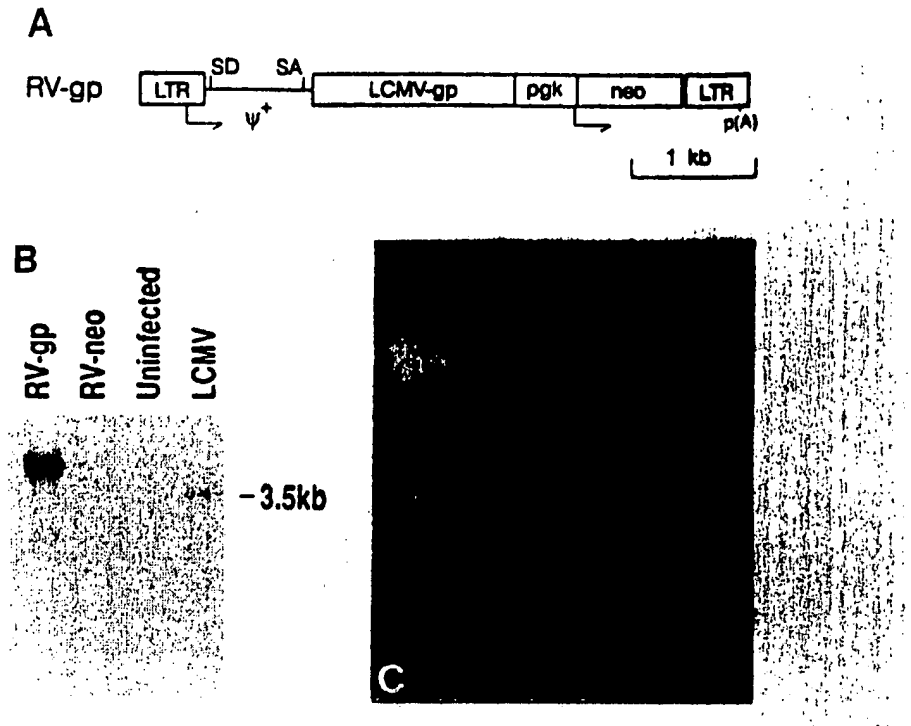

FIGURE 1. Expression of LCMV-gp in fibroblasts transduced with RV-gp. *A*, Structure of RV-gp. Arrows indicate long terminal repeats and phosphoglycerate kinase (pgk) promoters utilized for expression of the LCMV-gp cDNA and the *neo* gene, respectively. SD, splice donor site; SA, splice acceptor site; ψ^+, extended packaging region for high viral titer. *B*, Gp-specific RNA is detected by Northern analysis. Total RNA from NIH 3T3 cells transduced with RV-gp or RV-neo, uninfected NIH 3T3 cells, and LCMV-infected NIH 3T3 cells were hybridized with an LCMV-gp-specific probe. *c*, RNA gel stained with ethidium bromide confirmed that equal amounts of RNA were loaded in each lane.

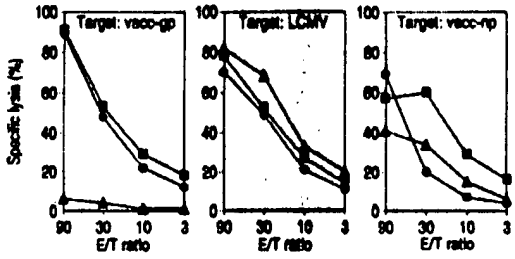

FIGURE 2. LCMV-gp-specific tolerance occurs in chimeric animals reconstituted with bone marrow transduced with RV-gp. Absence of LCMV-gp specific cytotoxic T cells in RV-gp chimeric mice. RIP-gp mice reconstituted with RV-neo (circles) and RV-gp (triangles) transduced bone marrow and control C57BL/6 mice (squares) were infected with LCMV. Spleen cells were assayed for cytotoxic function in a standard chromium release assay with LCMV-infected fibroblasts (MC57G), fibroblasts infected with a vaccinia recombinant virus that expresses LCMV-gp, or a vaccinia recombinant virus that expresses LCMV-np. Less than 5% specific lysis was detected using targets infected with control vaccinia virus alone (data not shown).

peripheral T cell repertoire, but a noteable absence of transgenic $CD8^+$ T cells was observed.

Spleen cells from chimeric animals reconstituted with TCR transgenic bone marrow TCR (RV-gp) and TCR (RV-neo) were also used in a proliferation assay. LCMV-gp-specific proliferative responses remained undetectable in animals reconstituted with TCR (RV-gp), whereas LCMV-specific proliferation was found using T cells from control TCR transgenic mice and control TCR (RV-neo) chimeric mice (Fig. 3*B*). Taken together, these studies demonstrated that LCMV-gp-specific tolerance is induced primarily by clonal deletion in animals reconstituted with RV-gp transduced bone marrow.

To examine whether the retrovirus was detectable in RV-neo and RV-gp chimeric mice, DNA was extracted from various tissues. PCR analysis was done using neomycin-specific primers, and Southern blot analysis of amplified DNA using a neomycin-specific probe demonstrated that the retroviral DNA was present in the bone marrow, thymus, spleen, and lymph nodes from chimeric mice (Fig. 4). Retroviral DNA was consistently detected in primary and secondary lymphoid organs of several RV-gp and RV-neo chimeric mice that had been reconstituted with RV-gp and RV-neo transduced bone marrow for 10 wk to 5 mo.

The most important question, however, was whether retroviral-mediated gene therapy could prevent autoimmunity in vivo. RV-gp and RV-neo transduced donor bone marrow were used to reconstitute transgenic RIP-gp host animals. Chimeric animals and control RIP-gp were infected with LCMV and the glucose levels were monitored. RIP-gp animals became hyperglycemic within 10 days after infection with LCMV as previously reported (5). Similarly, all RIP-gp (RV-neo) chimeric mice (6/6) developed overt hyperglycemia, while none of the RIP-gp (RV-gp) chimeric animals (0/14) developed hyperglycemia (Fig. 5 and Table I). Therefore, using this in vivo system, autoimmunity may be prevented by retroviral-mediated therapy.

Discussion

Several approaches have been taken to control T cell-mediated autoimmune diseases (30). Using animal models, treatment with anti-CD3 (31), anti-CD4, or anti-CD8 mAbs (24, 32, 33) have abrogated disease. The current clinical approach is to control the pathogenic T cells by using immunomodulatory drugs. However, these treatments are accompanied by other side effects as well as general immunosuppression, and therefore an approach that is directed specifically against the pathogenic T cells would be desirable.

Many studies have demonstrated that the induction of T cell tolerance specific for the target self Ag have resulted in the prevention

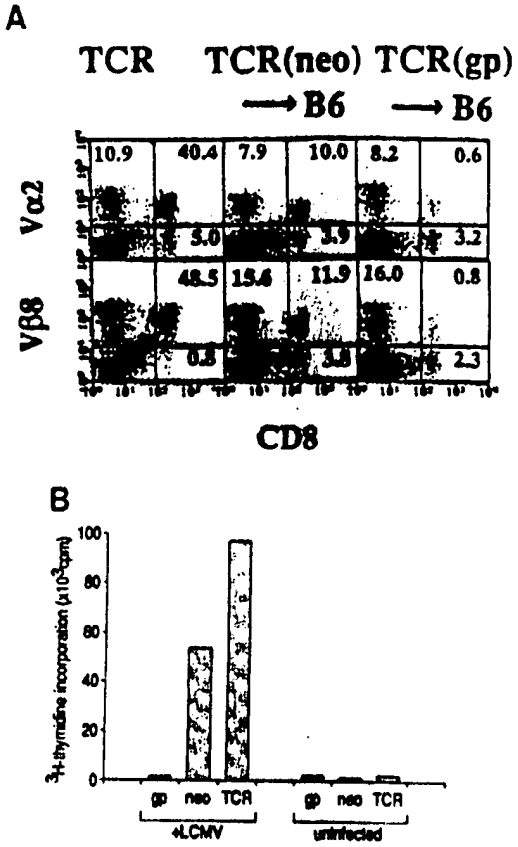

FIGURE 3. *A*, LCMV-gp-specific T cells are absent in the peripheral T cell repertoire. Two-parameter flow cytometry with either mAbs B20.1 (Vα2) or KJ16 (Vβ8.1, 8.2) and CD8 were used to detect transgenic TCR specific for LCMV-gp presented in the context of H-2D^b. Control TCR transgenic mice and chimeras reconstituted with RV-neo or RV-gp transduced TCR transgenic bone marrow are shown. *B*, LCMV-gp-specific proliferation is absent in chimeric mice reconstituted with TCR transgenic bone marrow transduced with RV-gp. Spleen cells from TCR transgenic mice or TCR bone marrow chimeric mice transduced with RV-gp and RV-neo were examined for a primary proliferative response to LCMV by cocultivation with LCMV-infected or uninfected peritoneal macrophages. SD from triplicate samples was <15% of mean. Chimeric animals were generated as described and tested 10 to 12 wk after reconstitution. Data are representative of animals from 4 to 6 independent experiments.

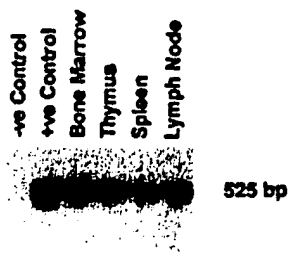

FIGURE 4. Retroviral DNA is detectable in RV-gp chimeric animals. DNA from various tissues was amplified by PCR using neomycin-specific primers, and analyzed by Southern blot with a neomycin-specific probe. Positive control is tail DNA from a gene-deficient mouse that has two copies of the neomycin gene, and negative control is tail DNA from a C57Bl/6 mouse. Similar findings have been shown for several chimeric mice generated using either RV-neo or RV-gp.

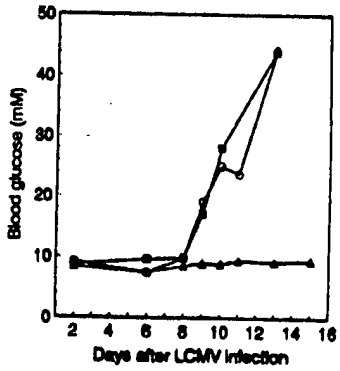

FIGURE 5. Hyperglycemia does not occur in bone marrow chimeric animals expressing RV-gp. RIP-gp transgenic mice were used as controls (squares) or bone marrow chimeras RIP-gp(RV-neo) (circles) or RIP-gp (RV-gp) (triangles) were infected with LCMV. RIP-gp (RV-gp) chimeras were monitored for 40 days, and hyperglycemia was not detected. Chimeric animals were generated as described and assayed after 3 to 5 mo.

Table I. *Autoimmunity does not occur in transgenic mice tolerized with RV-gp*

Mice	N	Hyperglycemia[a]	Day of Onset
RIP-gp	6	6	9
RIP-gp (RV-neo)	6	6	9
RIP-gp (RV-gp)	14	0	

[a] All hyperglycemic mice reached nonfasting glucose levels of a minimum of 20 mM.

of disease. Strategies that have been used involve transplanting the affected organ in the thymus to induce central tolerance (34, 35) or tolerizing autoreactive T cells by exposure to high amounts of the target self peptide or autoantigen in neonatal or adult mice (36–41). Vaccination against the autoantigen (42) or the agent that induces autoimmunity (37) have also reduced the incidence of autoimmunity in treated animals.

Other approaches to control autoimmunity have been based on the observation that the disease may be mediated by T cells that use a predominant TCR variable (V) region (4, 43–46). This finding has lead to therapies that control disease by using mAbs specific for the predominant TCR V regions (47), or treatment with peptides specific for a specific V region or autoreactive T cell lines to induce controlling cell populations (39, 48–52). However, these therapies are dependent on the presence of autoreactive T cell populations that utilize a predominant V region, which may not always exist (53–55).

In this study, we report an alternative approach by demonstrating that bone marrow expressing a self Ag through RV transduction can induce specific T cell tolerance and prevent autoimmunity in vivo. Although the target autoantigen must be defined, the advantage of this approach is that the dominant epitopes presented by a given MHC type does not need to be predetermined, and the requirement for preferential V region usage does not exist. Retroviral-mediated gene therapy is attractive because long term T cell tolerance may be induced against a specific autoantigen, thereby leaving an essentially normal functional T cell repertoire in the host. One limitation is that this model is directed towards a single transgenic autoantigen, and the pathogenesis of disease may involve several target autoantigens. Therefore, future studies using this system will examine whether t lerance may be directed towards multiple autoantigens, and will establish modified protocols

to determine the least invasive procedures necessary to induce central or peripheral tolerance.

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